



Avian Feeding Preferences of *Culex pipiens* and *Culiseta* spp. Along an Urban-to-Wild Gradient in Northern Spain

Mikel A. González¹, Sean W. Prosser², Luis M. Hernández-Triana³, Pedro M. Alarcón-Elbal⁴, Fatima Goiri¹, Sergio López⁵, Ignacio Ruiz-Arrondo⁶, Paul D. N. Hebert² and Ana L. García-Pérez^{1*}

¹ Department of Animal Health, NEIKER-Instituto Vasco de Investigación y Desarrollo Agrario, Basque Research and Technology Alliance (BRTA), Derio, Spain, ² Centre of Biodiversity Genomics, Biodiversity Institute of Ontario, University of Guelph, Guelph, ON, Canada, ³ Wildlife Zoonoses and Vector-Borne Diseases Research Group, Virology Department-Animal and Plant Health Agency, Addlestone, United Kingdom, ⁴ Universidad Agroforestal Fernando Arturo de Meriño (UAFAM), Jarabacoa, Dominican Republic, ⁵ Department of Biological Chemistry, Institute of Advanced Chemistry of Catalonia, CSIC, Barcelona, Spain, ⁶ Center for Rickettsiosis and Arthropod-Borne Diseases, Hospital Universitario San Pedro-CIBIR, Logroño, Spain

OPEN ACCESS

Edited by:

Jenny C. Dunn,
University of Lincoln, United Kingdom

Reviewed by:

Nathan Daniel Burkett-Cadena,
University of Florida, United States
Miguel Moreno-García,
Secretaría de Salud, Mexico
Andrea Egizi,
Monmouth County Mosquito Control,
United States

*Correspondence:

Ana L. García-Pérez
agarcia@neiker.eus

Specialty section:

This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 02 June 2020

Accepted: 24 September 2020

Published: 15 October 2020

Citation:

González MA, Prosser SW, Hernández-Triana LM, Alarcón-Elbal PM, Goiri F, López S, Ruiz-Arrondo I, Hebert PDN and García-Pérez AL (2020) Avian Feeding Preferences of *Culex pipiens* and *Culiseta* spp. Along an Urban-to-Wild Gradient in Northern Spain. *Front. Ecol. Evol.* 8:568835. doi: 10.3389/fevo.2020.568835

Mosquitoes (*Diptera*: *Culicidae*) are regarded as annoying biting pests and vectors of disease-causing agents to humans and other vertebrates worldwide. Factors that affect their distribution and host choice are not well understood. Here, we assessed the species abundance, community composition, and feeding patterns of mosquitoes in an urban-to-wild habitat gradient in northern Spain. Adult mosquitoes from four habitats (urban, periurban, rural, and wild) were collected by aspiration from mid-July to mid-September, 2019. Thirteen species were represented among the 268 specimens (132 females and 136 males) trapped, including six new records reported for the first time in the region. *Culex pipiens* was the most abundant species in all habitats except in the wild, where *Culex territans* was dominant. The highest mosquito diversity was recorded in the wild habitat [species richness (S) = 10 and Shannon/Margalef-Diversity Indices (H'/MI = 1.51/1.36)] and the lowest in the urban habitat (S = 3; H'/MI = 0.24/0.41). Blood-engorged specimens ($n = 65$) represented 49.2% of the total female collections. Eighty percent of the blood-meals ($n = 52$) were successfully identified based on cytochrome c oxidase I subunit (COI) DNA barcoding. Nine species of birds were identified in blood meals from the three ecological forms of *Cx. pipiens* ($n = 48$), *Culiseta fumipennis* ($n = 3$), and *Culiseta morsitans* ($n = 1$) collected along the four sampling habitats. Four dominant bird species were recorded in *Cx. pipiens*, i.e., *Parus major* (35.4%), *Turdus merula* (18.7%), *Pica pica* (18.7%), and *Passer domesticus* (10.4%). Despite the availability of dog and human hosts in the sampling sites located in the urban habitat, *Cx. pipiens* seemed to have a preference to feed on birds. *Culiseta fumipennis* blood-meal host records are reported for first time in Europe. These findings on mosquito blood-feeding preferences and habitat community changes will help to better understand vector-host associations and pathogen transmission paths.

Keywords: *Culex pipiens*, *Culiseta*, blood meals, avian hosts, cytochrome c oxidase I, biodiversity, urban-wild gradient

INTRODUCTION

Mosquitoes (*Diptera: Culicidae*) are recognized as the most important arthropod group negatively impacting human and animal health worldwide. Numerous mosquito species serve as enzootic, bridge, and/or epidemic vectors of human pathogens. Although tropical countries are more exposed to mosquito-borne diseases, Europe is experiencing an increasing number of human cases. In southern Europe, local autochthonous transmission of important arbovirus diseases has been recorded [dengue virus (DENV), Chikungunya virus (CHIKV), and Zika virus (ZIKV)]. Other arboviruses [West Nile virus (WNV); Usutu virus (USUV); Sindbis virus (SINV); Tahyna virus (TAHV); Batai virus (BATV); Inkoo virus (INKV); and Snowshoe Hare virus (SSHV)] but also protozoans (*Plasmodium* spp.) and nematodes (*Dirofilaria* spp.) have been notified in the continent with variable distribution and effect on humans (Calzolari, 2016). Specifically in Spain, WNV, USUV, and DENV viruses have been detected in native and non-native mosquito species (Vázquez et al., 2011; Aranda et al., 2018). Zoonotic parasitic diseases, such as the canine heartworm and other filarioid nematodes, have also been found in native mosquito species (Bravo-Barriga et al., 2016). Protozoan parasites of avian species are also prominent in Spain, i.e., *Haemoproteus* and *Plasmodium* are commonly found in mosquitoes fed on birds (Gutiérrez-López et al., 2016; Martínez-de la Puente et al., 2016).

Among native mosquito species, the genus *Culex* has deserved great attention, due to their biting nuisance and potential as bridge vector for different pathogens (Vázquez et al., 2011; Martínez-de la Puente et al., 2016; Brugman et al., 2018). Mosquitoes of the *Culex pipiens* complex have a cosmopolitan distribution, and include several species, subspecies, forms, races, physiological variants, or biotypes (Becker et al., 2012). No consensus exists on the taxonomic status of the members of this complex. In Europe, *Cx. pipiens* is presented into three intraspecific forms: *Cx. pipiens molestus*, *Cx. pipiens pipiens*, and *Cx. pipiens* hybrids (Brugman et al., 2018). Hybrids between *Cx. pipiens* and *Cx. quinquefasciatus* have also been reported from the Mediterranean Basin (Shaikevich et al., 2016). Both *Cx. pipiens* forms (*pipiens* and *molestus*) and their hybrids are present in the Iberian Peninsula (Martínez-de la Puente et al., 2016). *Cx. pipiens* has a good tolerance to human-altered environments and is an opportunistic feeder on birds and mammals, including humans (Brugman et al., 2018). Another native genus, namely *Culiseta*, includes some species that have been moderately implicated in the transmission of important arboviruses, such as USUV and WNV, in Europe (Martinet et al., 2019). Nevertheless, up to date no report of virus transmission has been noticed in Spanish mainland.

Abbreviations: BATV, Batai virus; CHIKV, Chikungunya virus; COI, Cytochrome c Oxidase Subunit 1; DENV, Dengue virus; DOI, Digital object identifier; H', Shannon-Wiener Diversity Index; INKV, Inkoo virus; ITS-2, Internal Transcribed Spacer 2; MI, Margalef Diversity Index; OTU, operational taxonomic units; SINV, Sindbis virus; S, Species richness; SSHV, Snowshoe Hare virus; TAHV, Tahyna virus; UMI, Universal molecular identifiers; USUV, Usutu virus; USUV, Usutu virus; WNV, West Nile virus; ZIKV, Zika virus.

Mosquitoes inhabit diverse types of habitats in urban, periurban, and wild environments, where they undertake host-seeking activity and search for suitable substrates for the development of their progeny. Several factors are involved in the mechanisms that affect spatial-temporal distribution and host selection. The urban-to-wild gradient is composed of distinct environmental habitats that influence distribution, diversity, and abundance of mosquito species (Ferraguti et al., 2016). In Europe, urbanization processes created suitable habitats for a small number of anthropophilic species, mostly *Cx. pipiens* (Becker et al., 2012), and *Aedes albopictus* (Li et al., 2014). Conversely, permanent water bodies in natural environments serve as favorable breeding sites for a wide range of species, such as *Anopheles maculipennis* s.l., *Aedes vexans*, *Aedes sticticus*, *Aedes caspius*, and *Aedes detritus* (Medlock and Vaux, 2015). The elucidation of how mosquito communities change along urban-to-wild habitats provides valuable information to characterize the environmental and ecological processes that significantly define the mosquito populations and host-related interactions.

Feeding patterns of hematophagous arthropods are also a critical component of the ecological cycles of pathogen transmission. Identifying the host affinity of mosquito vector species under field conditions is particularly important given the role of wild and domestic animals as reservoir hosts of several important arboviruses (Failloux et al., 2017). In addition, since not all avian species are competent hosts of arboviruses (Kilpatrick et al., 2007), the identification of blood meals is a first step for the detection of key reservoir species. Consequently, an enhanced understanding of the factors involved in the distribution and host-choice of mosquitoes, particularly disease vectors, would improve the efficiency of surveillance and control programs (Ferraguti et al., 2016).

Mosquito surveillance and screening efforts have been focused on the southern and eastern regions of Spain (Muñoz et al., 2011; Ferraguti et al., 2016; Martínez-de la Puente et al., 2016) while the northern regions have been comparatively overlooked due to their less favorable climatic conditions (harsh winters and short summers) for the proliferation of mosquitoes, bites and their diseases (Eritja et al., 2005; Ruiz-Arrondo et al., 2019). However, climate change and anthropogenic factors are beginning to affect the distribution and incidence of mosquito vectors and their pathogens, an example being the recent arrival and establishment of the exotic species *Ae. albopictus* (Goiri et al., 2020) and *Ae. japonicus* in northern Spain (Eritja et al., 2019).

The main goal of the present study was to ascertain the species diversity including the screening of potential exotic species, abundance, and feeding patterns of mosquito species along an urban-to-wild gradient in northern Spain. In addition, we determined the success of identification of female mosquitoes based on digestion status, and the gonotrophic stages distribution. Finally, the efficacy of active suction trapping as monitoring tool is discussed.

MATERIALS AND METHODS

Study Area and Sampling Sites

This study was conducted in the municipality of Vitoria-Gasteiz (ca. 260,000 inhabitants), capital of the Autonomous Community of the Basque Country (northern Spain). Four types of habitats, located 1.0, 2.5, 4.5, and 12.0 km from the city center, were selected following a gradient from a high-anthropogenic site (urban) to a highly conserved natural site (wild). The human population density for each habitat was an estimation calculated from demographic data from 2018 (Gobierno Vasco-Eusko Jaurlaritza, 2019) and the final selection of the study site was based on environmental characteristics (Figure 1A). The urban habitat (42.853397, -2.682756, and ca. 860 habitants/km²) was a densely populated area with buildings, schools, and multiple gardens containing hedges of *Prunus cerasifera* and *Chamaecyparis lawsoniana*, and several tree species, including *Aesculus hippocastanum*, *Magnolia grandiflora*, *Liriodendron tulipifera*, and *Paulownia tomentosa*. The periurban habitat (42.855776, -2.710002, and ca. 150 habitants/km²) consisted of an industrial environment mixed with buildings, wastelands and rows of *Quercus ilex* and *Fraxinus excelsior*. Both urban and periurban settings had sewer systems in the vicinity. The rural habitat (42.841991, -2.717480, and ca. 40 habitants/km²) comprised of a green patch (with paths, small lagoons, and diverse vegetation) surrounded by country homes and agricultural fields. The wild habitat (42.890751, -2.532650) where human activities were very limited or non-existent, was located in an ornithological park at the edge of a large water reservoir (swamp) which contained aquatic plants, thick vegetation, grasslands, and small woodlands.

Mosquito Collection and Host Counting

We employed a battery-powered Prokopack aspirator (John W. Hock, Florida, United States) to collect outdoor-resting mosquitoes across the four habitats. This quiet, low-cost, and easy handling aspirator allowed working in both open and densely vegetated areas without disturbing or attracting attention of humans and animals. Collections were conducted between 9:00 a.m. and 12:00 p.m. along a 100 m line transect, and lasted approximately 30 min per habitat. Each habitat was sampled fortnightly from mid-July to mid-September 2019, giving a total of five sampling periods. Transects were selected according to the presence of natural resting sites, such as humid and shadow shelters (tree-lines, hedgerows) and corners in concrete walls. Insect collection was carried out by passing the aspirator up and down and from side to side, so that the entire surface was sampled. Insects escaping from their resting place were also collected by aspirating them out of the air. Collection cups that became full or clogged were replaced as necessary during each collection event. Immediately after collection, the cups were stored at -30°C until further taxonomical and molecular analyses.

The number of hosts encountered in each sampling transect was scored concurrently to the mosquito aspiration along each sampling transect. In the case of mammals (i.e., humans and

dogs), count was done at the beginning, middle and end of each transect, during 5-min point counts with a limited observation radius (30 m), unless the features of the landscape minimized the visual capacity of the operator. For avian species, counting was performed in a similar way by determining the number of individuals that were seen either walking, perched on vegetation/trees or took off after the walking activity of the operator within the visual radius (30 m). In this regard, no determination was done at species level. Those birds flying up in the sky were excluded from the counting (Figure 1B).

Morphological Identification

Prior to taxonomical identification, specimens were separated by sex, and females sorted by their physiological status based on abdomen condition (blood-fed, gravid, and unfed). Species identification was done using appropriate taxonomic keys (Schaffner et al., 2001; Becker et al., 2010) based on morphological features of females and male genitalia. In order to identify blood-meal host species, abdomens of blood-engorged females were removed and individually stored in 2 ml screw-top vials after being classified according to the scale of Sella (II-VII) by observation of the degree of digestion of the blood and developing eggs (gravid stage = Sella stage VII) (Martínez-de la Puente et al., 2013).

Molecular Identification of Blood-Fed Mosquitoes and Their Hosts

Cytochrome c oxidase I subunit (COI) DNA barcoding was used to identify damaged or morphologically indistinguishable mosquito specimens ($n = 2$), as well as vertebrate host species of gravid ($n = 11$), and blood fed females ($n = 65$) at the Centre for Biodiversity Genomics, University of Guelph (Guelph, ON, Canada). Standard barcoding methods were used for mosquito identification (Hebert et al., 2003) while a modified version that accounts for DNA degradation, as well as the possibility of mixed feeding, was used for blood-meal vertebrate host identification. In all cases, abdomens were removed using sterile forceps and placed individually in 2-ml screw-tubes. DNA extraction was performed by immersing the abdomen in a lysis buffer containing proteinase-K followed by incubation at 56°C for 18 h. Blood-engorged abdomens were purposefully disrupted during transfer into screw-tubes to facilitate DNA extraction from the blood meal. DNA purification employed a glass fiber-based bind-wash-elute method (Ivanova et al., 2006). Briefly, the lysate was mixed with two volumes (120 µL) and a binding mix, and then applied to a well of a 96-well silica membrane plate. The plate was centrifuged at 5000 g for 5 min, and washed two times. Traces of wash buffer were removed by incubating the plate at 56°C for 30 min, after which 40 µL of elution buffer was applied directly to the membrane and allowed to incubate at room temperature for 1 min. DNA was eluted from the membrane into a clean plate via centrifugation at 5000 g for 5 min.

For mosquito identification, the entire barcode region of COI was amplified using the primers C_LepFolF + C_LepFolR (Hernández-Triana et al., 2014) and the products were used directly for Sanger sequencing (Hajibabaei et al., 2006). For

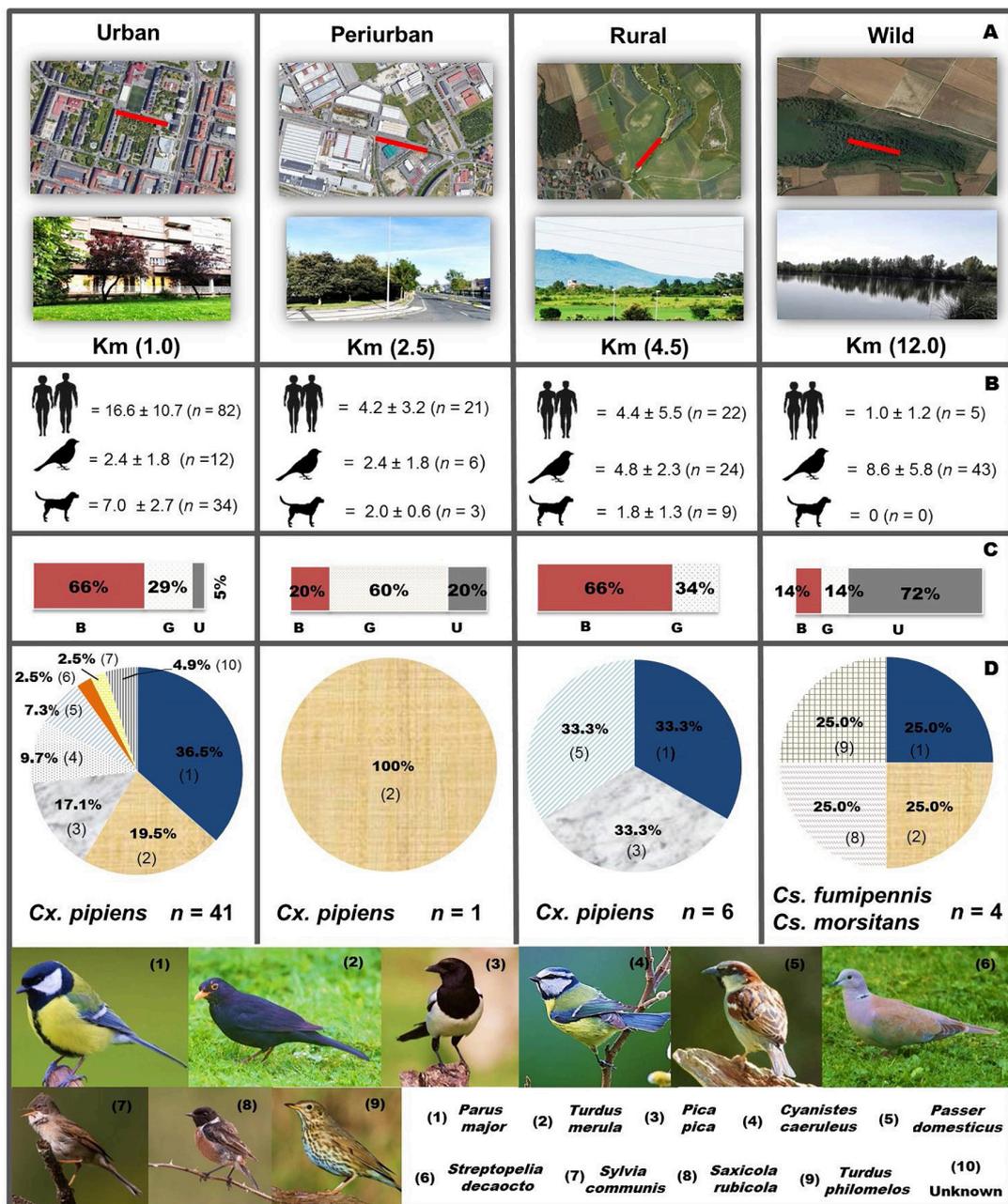


FIGURE 1 | Summary of sampling sites and host blood-meal sources for *Cx. pipiens* and *Culiseta* spp. **(A)** Sampling habitats gradient (urban, periurban, rural, and wild) with diminishing degree of anthropogenic modification (NASA Google Earth images). Red continuous line within the colored image denotes the 100 m linear transect sampling area. The images below show a general view of the sampled sites along with the distance (kilometers) from the city center. **(B)** Mean (\pm SEM) host abundance of humans, birds and dogs (n = total host abundance) recorded across sampling period. **(C)** Proportion of blood-engorged (B), gravid (G), and unfed (U) females in each habitat. **(D)** Host blood meal sources for mosquitoes collected (numbers in parenthesis refer to host species showed below; n = successful blood meals identifications).

blood meal analysis, PCR amplification consisted of a two-stage fusion approach: the first step amplified a short region of the COI barcode using primers specific to vertebrates (C_BloodmealF1_t1 + Mod.Mam.R_t1) (Estrada-Franco et al., submitted), while the second step added sample-specific universal molecular identifiers (UMI) and Ion

Torrent sequencing adapters. Specific conditions of PCR, thermocycling, and purification procedures are detailed in Estrada-Franco et al. (submitted). Briefly, the PCR reaction (12.5 μ L) consisted of 2.0 μ L of Hyclone ultra-pure water (Thermo Scientific), 1.25 μ L of 10X Platinum Taq buffer (Invitrogen), 0.625 μ L of 50 mM MgCl₂ (Invitrogen), 0.125 μ L

of each primer cocktail (10 μ M), 0.0625 μ L of 10 mM dNTP (KAPA Biosystems), 0.060 μ L of 5 U/IL Platinum Taq DNA Polymerase (Invitrogen), 6.25 μ L of 10% D-(+)-trehalose dihydrate (Fluka Analytical), and 2 μ L of DNA. The forward primer cocktail contained the primers BloodmealF1_t1 (TGTA AACGACGGCCAGTACCACWATTATTAAYATAAAR CCMC), BloodmealF2_t1 (TGTA AACGACGGCCAGTACTAC AGCAATTAACATAAAACCMC), while the reverse primer cocktail contained the primers VR1_t1 (CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCAAAG AATCA), VR1d_t1 (CAGGAAACAGCTATGACTAGACTTCTG GGTGGCCRAARAAYCA), and VR1i_t1 (CAGGAAACAGCTATGACTAGACTTCTGGGTGICCIAAIAA ICA). The forward primers were designed to bind to vertebrate – but not mosquito – DNA, so that only vertebrate blood meal host DNA is amplified. These primers targeted a 185 bp fragment of the COI barcode region, allowing them to amplify highly degraded blood meal DNA. The thermocycling profile consisted of initial denaturation at 95°C for 2 min, followed by 60 cycles of 95°C for 40 s, 56°C for 40 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. After confirming amplification on a 2% E-gel (Invitrogen), the PCR products were diluted two-fold with sterile water and used as a template for a second round of PCR. This second PCR (PCR2) served to index the PCR1 amplicons with IonXpress UMI tags and Ion Torrent sequencing adapters. The M13F and M13R tails of the PCR1 primers served as universal binding sites for PCR2. PCR2 reactions consisted of the same components as PCR1. The PCR2 thermocycling consisted of an initial denaturation at 95°C for 2 min, followed by 5 cycles of 95°C for 40 s, 45°C for 40 s, and 72°C for 30 s, and then 35 cycles of 95°C for 40 s, 51°C for 40 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR2 products were pooled in equal volumes and purified using SpeedBeads (Sigma Aldrich) by incubating 400 μ L of pooled PCR2 product with 400 μ L of beads. The DNA-bead mixture was incubated at room temperature for 8 min, after which the beads were collected on a magnetic rack. The bead pellet was washed three times with 1 mL of freshly prepared 80% ethanol and air dried for 10 min. DNA was released from the beads by mixing the pellet with 200 μ L of sterile water for 1 min at room temperature. The beads were collected on a magnetic rack and 180 μ L of the supernatant was transferred to a clean 1.5 mL tube. The purified library was quantified using a Qubit 2.0 fluorometer, adjusted to 26 pM with sterile water, and loaded onto an Ion Chef automated platform (Thermo Scientific) following manufacturer's instructions. Sequencing was performed using 400 bp chemistry on an Ion Torrent S5 (Thermo Scientific) using a 530 chip. Raw sequence reads were automatically demultiplexed following sequencing using the Torrent Browser sequencing software. The demultiplexed reads were further processed using standalone bioinformatics tools (see section “Bioinformatic, sequence information and statistical analysis” below for details). The efficacy of the technique to amplify both mammalian and avian hosts was validated prior to the analysis of the samples by testing the specificity of the primers by the inclusion of positive (DNA extracted from blood-fed insects known to have fed on certain vertebrate hosts), and negative controls. The amplified products

were sequenced to confirm that they originated from the target vertebrate species and not the insect.

Molecular Identification of Sibling Species and Forms

The identification of the members of the *Anopheles maculipennis* complex ($n = 5$) and blood fed/gravid *Cx. pipiens* (a total of 64 out of 76 gravid/blood-fed female mosquitoes submitted to the molecular methods described in the previous section) ecological forms were carried out from DNA extracted from the thorax of individual specimens in the Animal Health Department of NEIKER (Spain). In both cases, genomic DNA extraction was carried out by NZY Tissue gDNA isolation kit (NZYTech, Lisboa, Portugal). Identification of the specimens of the *An. maculipennis* species complex was carried out by a PCR-RFLP assay targeting polymorphisms in the Internal Transcribed Spacer 2 (ITS-2) of the ribosomal DNA (Vicente et al., 2011). Amplicons were digested first with *HhaI* and secondly with *HpaII* restriction enzymes (Invitrogen/Thermo Scientific, Vilnius, Lithuania). Identification of the different *Cx. pipiens* female forms (*Cx. pipiens pipiens*, *Cx. pipiens molestus*, and its hybrids) was performed by PCR amplification of the flanking region of the CQ11 microsatellite, following the protocol already described (Bahnck and Fonseca, 2006). Amplicons from both PCR methods were separated by 2% agarose gel electrophoresis using GelRed Nucleid Acid Stain (Biotium, Hayward, CA, United States) as staining solution, and with a 100 bp DNA ladder (Thermo Scientific, Vilnius, Lithuania) as a molecular weight marker.

Bioinformatic, Sequence Information and Statistical Analysis

Raw sequence reads were filtered (based on a minimum length of 100 bp and minimum quality of QV20) and cleaned (removal of adapter and primer tails) to ensure that only high-quality reads were included in the final dataset. Reads passing these filters were clustered into operational taxonomic units (OTUs) with 97% identity and a minimum of 10 reads per OTU. Each OTU sequence was compared using BLAST tool¹ and BOLD Systems², which are composed of global vertebrate COI sequences. Identifications were considered valid only if the query sequence matched a reference sequence with at least 95% identity with 100 bp of coverage between the queried sequence and the reference sequence. Furthermore, mosquito identifications were manually vetted in order to ensure that they conformed with local fauna sighting records.

Detailed specimen records and sequence information (including trace files) were uploaded to the Barcode of Life Database (BOLD-<http://www.boldsystems.org>) and can be found within the Working Group 1.4 Initiative “Human Pathogens and Zoonoses” container “MCBCS-Surveillance of mosquitoes and *Culicoides* in the Basque Country, Spain.” The Digital Object Identifier (DOI) for the publicly available projects in BOLD is [doi:dx.doi.org/10.5883/DS-MQBMBC](https://doi.org/10.5883/DS-MQBMBC). All generated

¹www.ncbi.nlm.nih.gov/genbank

²<http://www.boldsystems.org/index.php/>

sequences have been submitted to GenBank (accession numbers: MT519609-MT519682).

The diversity indices of Shannon and Margalef (H'/MI), and species richness (S) were used as a measure of mosquito community heterogeneity. Host counting was obtained from the sum of the five trapping periods. Both mean abundances of mosquitoes within habitats and gonotrophic stages were compared by non-parametric Kruskal–Wallis test followed by Mann–Whitney U test pairwise comparisons (adjusting significance levels). All tests were conducted with IBM SPSS statistics v 23.0 software package.

RESULTS

A total of 268 mosquitoes (132 females and 136 males; mean \pm SEM = 13.4 ± 2.9 specimens/habitat) belonging to four genera and 13 species were identified during the five sampling periods (Table 1). The highest number of mosquitoes was recorded in mid-August (Supplementary Table 1). Six new records (*Culiseta annulata*, *Cs. fumipennis*, *Cs. morsitans*, *Cx. territans*, *Cx. theileri*, and *Uranotaenia unguiculata*) are reported for first time in the Basque Country region. No exotic invasive mosquitoes were recorded in the study area.

Mosquito Community Composition and Abundance

Cx. pipiens was the most predominant species (200 specimens, 74.6% from the total collections), followed by *Cx. territans* (32,

11.9%), *Cs. longiareolata* (11, 4.1%), and other less frequent species (25, 9.3%; Table 1 and Figure 2A). *Cx. pipiens* was the only species collected along the four habitats and the most predominant in all sampled settings except in the wild habitat, where *Cx. territans* was the dominant species. Despite mosquito abundance being greater in the urban habitat, the difference was not significant ($\chi^2 = 6.4$, $df = 3$, and $p = 0.094$). Higher mosquito S and biodiversity was recorded in the wild habitat ($S = 10$, $H'/MI = 1.51/1.36$) compared to the other habitats ($S \leq 4$; $H'/MI \leq 0.40/0.75$; Table 1).

Molecular identification of blood-fed/gravid females by means of PCR (COI gene) and sequencing, confirmed the identity of *Cx. pipiens* specimens, thus this species is referred in the text as *Cx. pipiens* (100% match identity to bank identification number in BOLD: AAA4751, accession number GU908075). Note that molecular identification allowed the separation of *Cx. torrentium* females from the sibling *Cx. pipiens* females.

The pattern of *Cx. pipiens* ecological forms was successfully determined in 89.1% (57/64) of the blood-fed/gravid *Cx. pipiens* analyzed, showing the presence of the three ecological forms in different proportions [*Cx. pipiens pipiens* ($n = 50$; 87.7%; in urban, periurban and rural habitats); *Cx. pipiens molestus* ($n = 3$; 5.3%; in urban and rural habitats); and *Cx. pipiens* hybrids ($n = 4$; 7.0%; solely in urban habitat)]. *Cx. pipiens pipiens* was predominant in all the habitats but the wild, in which no *Cx. pipiens* blood-fed specimens were collected.

Morphologically similar members of the subgenus *Culiseta* were separated into three species (*Cs. fumipennis* voucher specimen = 100% identity, accession number KM258139; *Cs.*

TABLE 1 | Species diversity of mosquitoes along an urban-to-wild gradient in northern Spain.

Species	Sampled habitats															
	Urban				Periurban				Rural				Wild			
	F	M	T	%	F	M	T	%	F	M	T	%	F	M	T	%
<i>Cx. pipiens</i>	77	36	113	94.2	7	42	49	90.7	5	12	17	85.0	4	17	21	28.4
<i>Cx. territans</i>	0	0	0	0	0	0	0	0	0	0	0	0	13	19	32	43.2
<i>Cs. longiareolata</i>	3	3	6	5.0	1	1	2	3.7	0	0	0	0	2	1	3	4.1
<i>Cs. fumipennis</i>	0	0	0	0	0	0	0	0	0	0	0	0	6	0	6	8.1
<i>An. maculipennis</i> s.s.	0	0	0	0	0	0	0	0	0	0	0	0	5	0	5	6.8
<i>Cx. hortensis</i>	0	1	1	0.8	1	1	2	3.7	1	0	1	5.0	0	0	0	0
<i>An. claviger</i> s.l.	0	0	0	0	1	0	1	1.9	0	0	0	0	0	2	2	2.7
<i>Cs. morsitans</i>	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	2.7
<i>Cx. theileri</i>	0	0	0	0	0	0	0	0	1	0	1	5.0	0	0	0	0
<i>An. plumbeus</i>	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1.4
<i>Cs. annulata</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1.4
<i>Cs. litorea</i>	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1.4
<i>Ur. unguiculata</i>	0	0	0	0	0	0	0	0	1	0	1	5.0	1	0	0	0
Total	80	40	120		10	44	54		8	12	20		34	40	74	
Species richness (S)	3				4				4				10			
Mean (SEM)	23.6 (6.4)				10.8 (35.4)				4.2 (1.3)				14.4 (6.2)			
Shannon-Index (H')	0.24				0.40				0.39				1.51			
Margalef-Index (MI)	0.41				0.75				0.66				1.36			

*F = females, M = Males, and T = total.

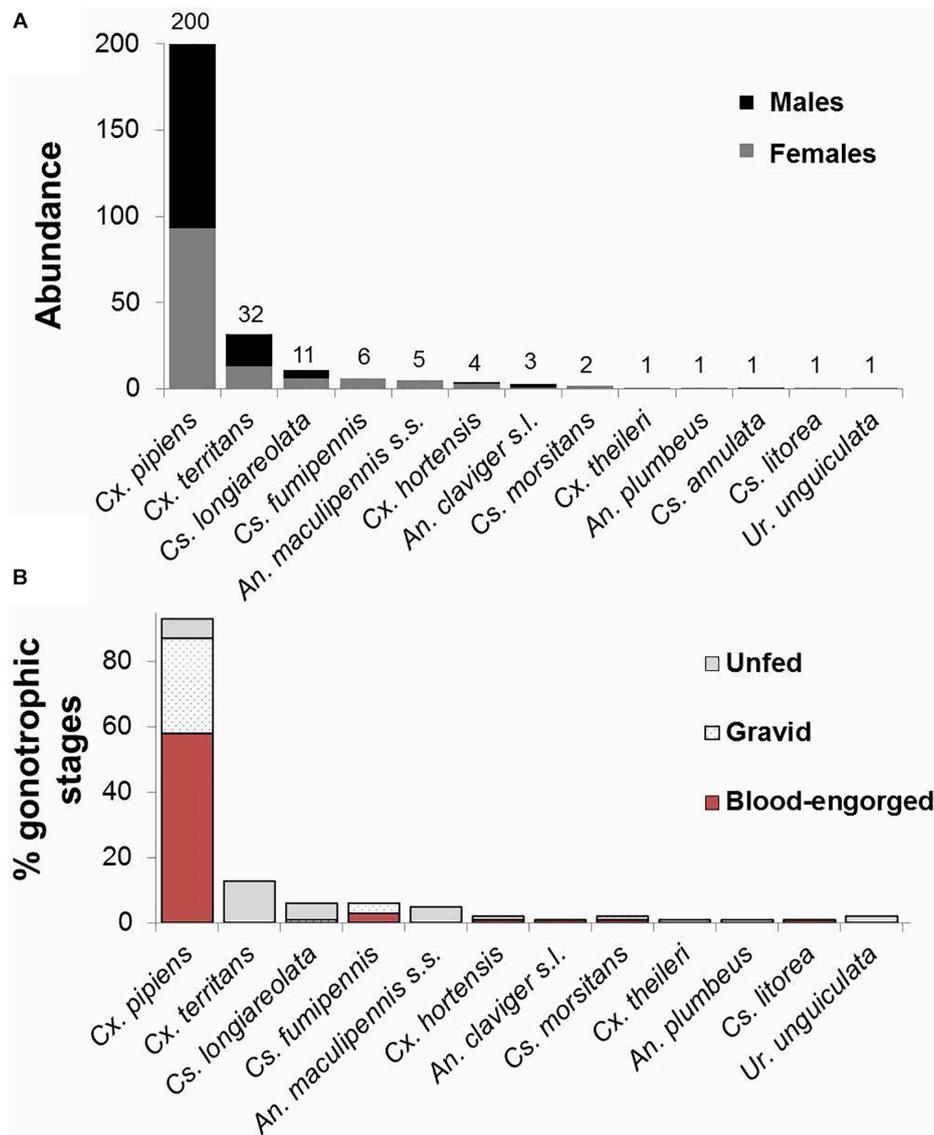


FIGURE 2 | Catches of mosquito species recorded from all the sampling settings. **(A)** Males and females. **(B)** Gonotrophic stages of females.

litorea = 98.8% identity, accession number MK402821; and *Cs. morsitans* = 99.7% identity, accession number KM258135).

Mosquitoes of the *Anopheles maculipennis* complex ($n = 5$) were identified as *An. maculipennis* sensu stricto (bands at 300 bp after *Hha*I digestion, and 200 bp after *Hpa*II digestion).

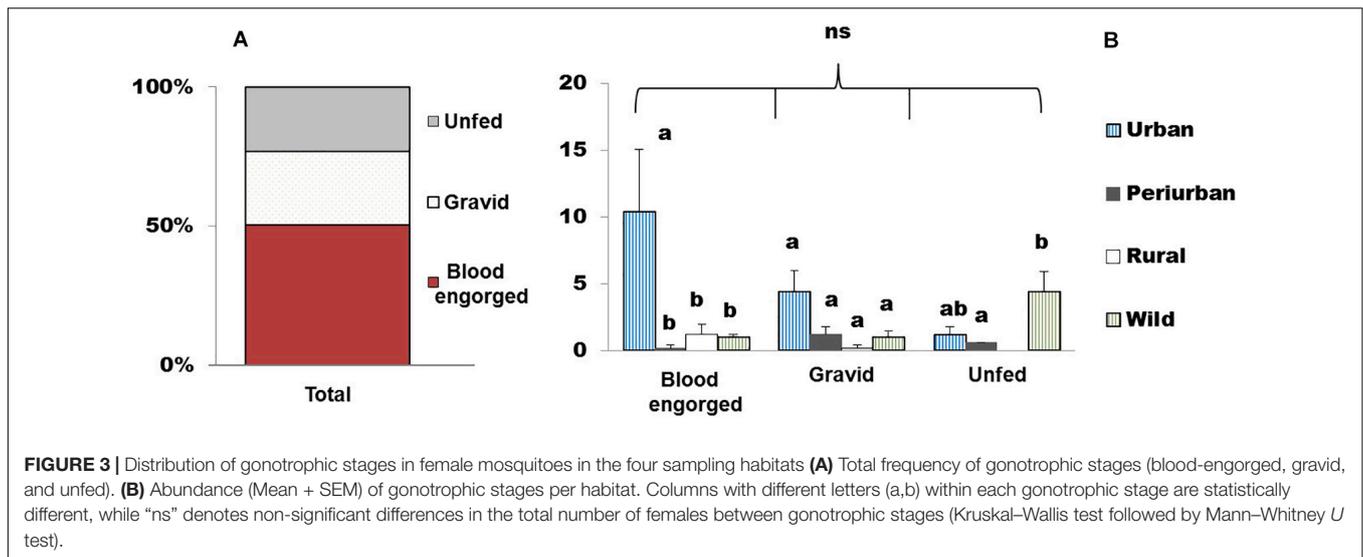
Analysis of Gonotrophic Stages

The distribution of the gonotrophic stage categories for each habitat and species is represented in **Figures 1C, 2B**, respectively. Overall, blood-engorged females represented the highest percentage of the catches, accounting for 49.2% (65/132) of the total (**Figure 3A**). However, no statistically significant differences were detected in the abundance of the three gonotrophic stages ($\chi^2 = 0.002$, $df = 2$, and $p = 1.2$) (**Figure 3A**). In contrast, significant differences were found in

the number of blood-fed ($\chi^2 = 10.64$, $df = 3$, and $p = 0.014$) and unfed females ($\chi^2 = 11.84$, $df = 3$, and $p = 0.008$) within habitats (**Supplementary Table 2** and **Figure 3B**). The statistical analysis revealed that significantly more blood-engorged females were aspirated in the urban habitat, while unfed individuals constituted the dominant gonotrophic stage in the wild habitat. Regarding gravid females, no differences were detected among sampling sites.

Host-Derived Blood-Meals Identification

Blood-meal host identification was successful for 80.0% ($n = 52$) of the 65 engorged females analyzed in this study. All the stages within the scale of Sella (II–VI) yielded high number of successful identifications regardless their blood-digestion degree [II = 9/13



(69.2%), III = 6/7 (85.7%), IV = 6/6 (100%), V = 20/22 (90.9%), and VI = 11/17 (64.7%)] while those scored as gravid stages (stage VII, $n = 11$) failed to produce sequences.

A total of 52 bird hosts of at least nine species were identified for the three blood-fed mosquito species. *Cx. pipiens* ($n = 48$) fed on the great tit (*Parus major*, 35.4%), blackbird (*Turdus merula*, 18.7%), magpie (*Pica pica*, 18.7%), house sparrow (*Passer domesticus*, 10.4%), blue tit (*Cyanistes caeruleus*, 8.3%), Euroasian collared dove (*Streptopelia decaocto*, 2.1%), whitethroat (*Sylvia communis*, 2.1%), and two unspecific bird species (one belonging to Order Accipitriformes, 95% sequence match) (Figure 1D).

Culiseta spp. ($n = 4$) fed upon the European stonechat *Saxicola rubicola*, the song thrush *Turdus philomelos* and *P. major* for *Cs. fumipennis*, and *T. merula* for *Cs. morsitans* (Figure 1D). Blood-engorged specimens from three other species (*Cx. hortensis*, *Cs. litorea*, and *An. claviger* s.l.) tested negative to amplification. Mixed host feeding was not detected in any of the analyzed specimens.

DISCUSSION

The results of this study revealed that *Cx. pipiens* mosquitoes were the dominant species in urban, periurban, and rural habitats, where they showed a preferred tendency to feed on avian hosts. This feeding pattern was clearly observed in the urban setting, where a higher number of blood-fed *Cx. pipiens* females were trapped. Taking into account the predominance of non-avian hosts (humans and dogs) in the urban environment, it is highly remarkable that the blood-feeding pattern recorded for *Cx. pipiens* was strongly biased to bird hosts. In contrast, the wild habitat did not yield blood-fed specimens of the two predominant *Culex* species (i.e., *Cx. pipiens* and *Cx. territans*), though blood-fed *Culiseta* spp. were demonstrated to have fed also on bird hosts.

In this study, neither human nor dog sequences were obtained in blood from *Cx. pipiens* mosquitoes from the urban habitats

despite the relative high abundance of these hosts (64% and 26%, respectively). Similarly, humans were highly present in periurban and rural habitats (70% and 40%, respectively). On the basis of relative host abundance measures and mosquito diet, our results suggest a prevailing affinity of *Cx. pipiens* to feed on avian hosts in these habitats, however, it is not possible to definitively prove that the mosquitoes were not feeding on certain mammalian hosts (i.e., a failure to detect a host could be due to DNA degradation, primer binding issues for certain taxa, etc.). In Europe, the ornithophilic preference of *Cx. pipiens* has been observed in some studies (Muñoz et al., 2012; Roiz et al., 2012; Gomes et al., 2013; Radrova et al., 2013; Brugman et al., 2017), however, other authors have also highlighted the importance of mammals as a relevant feeding source (Alcaide et al., 2009; Muñoz et al., 2011; Rizzoli et al., 2015; Börstler et al., 2016; Martínez-de la Puente et al., 2016). Both human and non-human vertebrates (i.e., *Capreolus capreolus*, *Sus scrofa*, *Lepus europaeus*, *Canis lupus familiaris*, *Felis silvestris*, *Equus ferus caballus*, *Bos taurus*, *Oryctolagus cuniculus*, *Rattus rattus*, and some reptiles) from *Cx. pipiens* have been reported in variable proportions depending on the study and regardless of the *pipiens* forms (Alcaide et al., 2009; Muñoz et al., 2011; Rizzoli et al., 2015; Börstler et al., 2016; Martínez-de la Puente et al., 2016). Five out of the eight species of birds identified in the blood-meals from the urban setting were included in the bird census undertaken by ornithologists in the same park (Ayuntamiento de Vitoria-Gasteiz, 2019). They noted *Columbia livia* and *P. domesticus* as the most frequent birds, followed by *Apus apus*, *P. pica*, *Cyanistes caeruleus*, *P. major*, *T. merula* and, to a lesser extent, three other species. This marked feeding tendency of *Cx. pipiens* to feed on *T. merula* and *P. domesticus* was also observed in other European studies (Muñoz et al., 2012; Roiz et al., 2012; Gomes et al., 2013; Rizzoli et al., 2015). It is noteworthy that synanthropic and abundant bird species such as the rock pigeon *C. livia* could have been underestimated, since their abundance in the urban setting did not reflect the proportion of blood meals taken, which is consistent with other studies carried

out in Europe and United States (Rizzoli et al., 2015; Kothera et al., 2020). Analyses of gravid/blood-fed *Cx. pipiens* showed that they fed mostly on birds regardless of the ecological form, which is in agreement with similar studies carried out in Spain, United Kingdom, and Portugal (Gomes et al., 2013; Martínez-de la Puente et al., 2016; Brugman et al., 2017). Consequently, mosquito populations that mostly feed on birds would have a larger capacity to amplify WNV due to the fact that humans and other mammals do not support viremia high enough to infect mosquitoes (Platt et al., 2007).

In the wild habitat, the few blood-meals recorded corresponded to bird hosts from *Cs. fumipennis* and *Cs. morsitans*, which is in agreement to the relative bird density observed (89% of visible host). According to literature, *Culiseta* species feed on birds and mammals, including humans (Brugman, 2016), but in United States they have also been found feeding on reptiles (Blosser et al., 2017). To best of our knowledge, the *Cs. fumipennis* blood-meal identification represents the first report of the feeding pattern of this species in Europe. In Germany and United Kingdom, *Cs. morsitans* have been recorded feeding on birds but also on mammals, including humans (Börstler et al., 2016; Brugman et al., 2017). Further studies are necessary to establish solid conclusions about the feeding preferences of both species.

According to our findings, the highest *S* was found when the distance was increased from the urban sampling site, with the wild site harboring the highest number of species. This relatively low *S* is tightly linked to the negative effect of the urbanization processes on the diversity of mosquito communities (Ferraguti et al., 2016). Non-disturbed wild habitats, in contrast, are constituted by a plethora of suitable heterogeneous microhabitats, leading to a favorable scenario for enhancing mosquito diversity. Environmental factors including land use, vegetation, and hydrological characteristics are known to affect mosquito abundance and community composition (Ferraguti et al., 2016). Among them, the spatial heterogeneity gradient is reported as one of the most important key indicators with relevant influence in mosquito species diversity and distribution (Overgaard et al., 2003; Leisnham et al., 2014). For instance, urbanized environments are organized in small patches (buildings, green areas, and streets) which are highly structured with many interfaces between them. Conversely, natural environments habitats are structured in larger patches and less interfaces, resulting in high species diversity. As stated by McDonnell and Pickett (1990), suburban areas are usually prone to congregate higher *S* and diversity. Indeed, we observed that fewer species were found in urbanized environments compared to rural/wild environments. However, the number of catches did not follow a well-defined pattern, as the highest abundance was observed in the anthropogenic habitat. This contrasts with previous reports (Ibañez-Justicia et al., 2015; Ferraguti et al., 2016; de Valdez, 2017; Honnen and Monaghan, 2017) but the collection method employed was different among surveys (i.e., CDC-mini light traps, BG-Sentinel traps baited with CO₂, and aspiration). One possible explanation to the observed discrepancies is that mosquitoes could be more concentrated on the sampling site of the urban setting where fewer resting sites were available to use compared to wild areas, which in turn

offer a wide variety of features for mosquito activity (Burkett-Cadena et al., 2013). These discrepancies are the result of the presence of substantial numbers of *Cx. pipiens* (mostly the *pipiens* form) and *Cs. longiareolata*, highly anthropophilic and container-breeding species. Both species find in urban areas their preferred oviposition sites, i.e., artificial habitats such as urban sewage and ground water systems (Bueno Mari, 2010), and other temporary water bodies (gardens, terraces, etc.). *Cx. pipiens* abundance decreased as the distance from the urban setting increased, being replaced by other species, such as *Cx. territans*, *Cs. fumipennis*, and *An. maculipennis* s.s. The lower abundance of mosquitoes in the natural habitat might have been caused by the presence of a dense vegetated barrier between the sampling transect and swamp land, which could have prevented the dispersion of mosquitoes from breeding sites. Indeed, mosquito community composition and habitat selection are highly variable among countries (Möhlmann et al., 2017). The combination of various collection methods would allow a better representation of the mosquito and host community fauna, however, the use of other routine traps, i.e., suction traps are inappropriate as they are subject to vandalism and logistic constraints in public urban/periurban areas.

Identification rates of host-derived blood-meals were not consistent with Sella's scale, since we did not observe a marked reduction in the amplification success as the degree of digestion in the blood increased (Martínez-de la Puente et al., 2013; Brugman et al., 2017; Santos et al., 2019). These discrepancies may be attributable to the technique implemented which has been improved for amplification of degraded DNA. In addition, the technique used did not allow the identification of host-derived blood meals from gravid females, thus limiting the determination of host-feeding patterns exclusively to specimens containing signs of blood on their abdomens.

Our study has demonstrated the effectiveness of the sampling performance of the Prokopack aspirator for collection of outdoor-mosquitoes in comparison to other superior models (i.e., CDC-BP aspirator) (Vazquez-Prokopec et al., 2009; Maia et al., 2011). The Prokopack aspirator is simple to use, easier to maneuver, cheap, and lightweight. In addition, aspiration yielded larger numbers of blood-engorged specimens when compared to other standard methods (Maia et al., 2011; Brugman et al., 2017; Santos et al., 2019), which makes this technique suitable for studies of host-feeding preferences and/or pathogen detection. Thus, captures of blood-fed females are usually scarce (0.5–3.0%) using CDC traps (Roiz et al., 2012; Radrova et al., 2013). Moreover, direct aspiration from mosquito resting sites provides a more representative information and estimation of physiological condition, richness, abundance, sex ratio, and age structure (Silver, 2008) in comparison to other trapping methods. Conversely, the main constraint is the damage inflicted on the specimens after being aspirated and retained in the trap mesh for a long time, which frequently hampers proper identification of females. The differences in the gonotrophic stage frequencies (i.e., proportion of blood meals taken by mosquitoes) of female mosquitoes among habitats may be influenced by multiple factors difficult to be analyzed, such as the composition, abundance, and availability of vertebrate hosts, which in turn is linked to the

landscape anthropization level and ecological conditions. Other variables such as collection methods (e.g., CDC traps baited with CO₂ capture preferentially host-seeking females) and availability of breeding and resting places with different accessibility (e.g., in the urban setting lower resting places were available compared to the wild) (Maia et al., 2011; Barrera et al., 2012; Muñoz et al., 2012; Ferraguti et al., 2016), could explain the high proportion of blood-fed and unfed mosquitoes in urban and wild habitats, respectively. For example, in our study collections might be biased toward mosquitoes fed on avian hosts as most sampling sites were bird resting sites (bushes, tree hedges, etc.). A more controlled design of the sampling sites selection and a more accurate host counting method might help to solve these issues.

Faunistic studies focused on mosquito communities in northern Spain are scarce and mostly devoted to the detection of those species with relevant medical interest (Ruiz-Arrondo et al., 2019; Goiri et al., 2020). Here, we report for the first time the presence of six new species in the region, increasing from 14 to 20 the total number of species present in the Basque Country (Bueno-Marí et al., 2012; González et al., 2015, 2020; Goiri et al., 2020), including new records of *Anopheles maculipennis* s.s., a major vector of several pathogens in Europe (Kampen et al., 2016). Interestingly, in nearby territories, only *An. atroparvus* had been identified within this species complex (Ruiz-Arrondo et al., 2019). This study also contributes to the differentiation of *Culiseta* sibling species by barcoding. The separation of some female species of *Culiseta* remains challenging by both DNA barcoding and morphological features (Ruiz-Arrondo et al., 2020). The female sequences obtained had enough genetic divergence to be separated into individual OTUs (data not shown).

In conclusion, *Cx. pipiens* was the most widespread species along the studied habitats according to the aspiration-based sampling methodology. *Cx. pipiens*, *Cs. fumipennis*, and *Cs. morsitans* exhibited an overwhelming affinity for avian hosts. These results on mosquito blood meals and community composition contribute to a better understanding on the transmission risk of pathogenic agents of medical and veterinary importance. The study also highlights the importance of implementing monitoring programs that include mosquito species and host-feeding surveillance but also vector ecology.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

REFERENCES

Alcaide, M., Rico, C., Ruiz, S., Soriguer, R., Muñoz, J., and Figuerola, J. (2009). Disentangling vector-borne transmission networks: a universal DNA barcoding method to identify vertebrate hosts from arthropod bloodmeals. *PLoS One* 4:e7092. doi: 10.1371/journal.pone.0007092

AUTHOR CONTRIBUTIONS

MG and AG-P conceived and planned the experiments. MG carried out the field experiments and morphological identification of mosquitoes. SL performed statistical analyses. SP, PH, and LH-T carried out the molecular analysis. FG made molecular identification of sibling species and forms. MG, PA-E, SL, and IR-A wrote the manuscript. AG-P and FG reviewed and edited the final version of the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

FUNDING

This work was funded by the Department of Economic Development and Infrastructures of the Basque Government. MG was beneficiary of a post-doctoral contract financed by Department of Education of the Basque Government and FG is beneficiary of a Ph.D. contract funded by Department of Economic Development and Infrastructures of the Basque Government (FPI-2019). Sequencing expenses were covered by the Research Excellence Fund (Canada).

ACKNOWLEDGMENTS

The authors thank “Unidad de Anillo Verde y Biodiversidad del Ayuntamiento de Vitoria-Gasteiz” and “Centro de Estudios Ambientales del Ayuntamiento de Vitoria-Gasteiz” for their approval and consent to perform this study at bird-relevant observation spots. Bird pictures were taken with a Canon (EOS-1Ds Mark II + 600 mm f4) by the ornithologist Mr. Brain Webster (Instituto Alavés de la Naturaleza) and used here under his consent and approval.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2020.568835/full#supplementary-material>

Supplementary Table 1 | Species of mosquitoes collected during the five sampling periods in each habitat (urban, periurban, rural, and wild).

Supplementary Table 2 | Gonotrophic stages (blood-engorged = B, gravid = G and unfed = U) of female mosquito species along the habitats sampled.

Aranda, C., Martínez, M. J., Montalvo, T., Eritja, R., Navero-Castillejos, J., Herreros, E., et al. (2018). Arbovirus surveillance: first dengue virus detection in local *Aedes albopictus* mosquitoes in Europe, Catalonia, Spain, 2015. *Euro. Surveill.* 23:1700837. doi: 10.2807/1560-7917

Ayuntamiento de Vitoria-Gasteiz (2019). *Ciencia Ciudadana-Programa Conservación-Aves Urbanas. Mapa de Observaciones de 2019*. Available

- online at: https://www.vitoria-gasteiz.org/wb021/was/contenidoAction.do?idioma=es&uid=u39e55291_14ac2d076fb__7f84 (accessed May 22, 2020).
- Bahnck, C. M., and Fonseca, D. M. (2006). Rapid assay to identify the two genetic forms of *Culex (Culex) pipiens* L. (Diptera: Culicidae) and hybrid populations. *Am. J. Trop. Med. Hyg.* 75, 251–255. doi: 10.4269/ajtmh.2006.75.2.0750251
- Barrera, R., Bingham, A. M., Hassan, H. K., Amador, M., and Mackay, A. J. (2012). Vertebrate hosts of *Aedes aegypti* and *Aedes mediiovittatus* (Diptera: Culicidae) in rural Puerto Rico. *J. Med. Entomol.* 49, 917–921. doi: 10.1603/me12046
- Becker, N., Jöst, A., and Weitzel, T. (2012). The *Culex pipiens* complex in Europe. *J. Am. Mosq. Control Assoc.* 28 (4 Suppl.), 53–67. doi: 10.2987/8756-971X-28.4s.53
- Becker, N., Petric, D., Zgomba, M., Boase, C., Madon, M., Dahl, C., et al. (2010). *Mosquitoes and Their Control*, 2nd Edn, Heidelberg: Springer.
- Blosser, E. M., Lord, C. C., Stenn, T., Acevedo, C., Hassan, H. K., Reeves, L. E., et al. (2017). Environmental drivers of seasonal patterns of host utilization by *Culiseta melanura* (Diptera: Culicidae) in Florida. *J. Med. Entomol.* 54, 1365–1374. doi: 10.1093/jme/tjx140
- Börstler, J., Jöst, H., Garms, R., Krüger, A., Tannich, E., Becker, N., et al. (2016). Host-feeding patterns of mosquito species in Germany. *Parasit. Vect.* 9:318. doi: 10.1186/s13071-016-1597-z
- Bravo-Barriga, D., Parreira, R., Almeida, A. P. G., Calado, M., Blanco-Ciudad, J., Serrano-Aguilera, F. J., et al. (2016). *Culex pipiens* as a potential vector for transmission of *Dirofilaria immitis* and other unclassified Filarioidea in Southwest Spain. *Vet. Parasitol.* 15, 173–180. doi:10.1016/j.vetpar.2016.04.030
- Brugman, V. (2016). *Host Selection and Feeding Preferences of Farm-Associated Mosquitoes (Diptera: Culicidae) in the United Kingdom*. Ph. D thesis, London School of Hygiene & Tropical Medicine, London.
- Brugman, V. A., Hernández-Triana, L. M., England, M. E., Medlock, J. M., Mertens, P. P. C., Logan, J. G., et al. (2017). Blood-feeding patterns of native mosquitoes and insights into their potential role as pathogen vectors in the Thames estuary region of the United Kingdom. *Parasit. Vect.* 10:163. doi: 10.1186/s13071-017-2098-4
- Brugman, V. A., Hernández-Triana, L. M., Medlock, J. M., Fooks, A. R., Carpenter, S., and Johnson, N. (2018). The role of *Culex pipiens* l. (Diptera: Culicidae) in virus transmission in Europe. *Int. J. Environ. Res. Public Health* 15:389. doi: 10.3390/ijerph15020389
- Bueno Marí, R. (2010). *Biocología, Diversidad e Interés Epidemiológico de los Culicidos Mediterráneos (Diptera: Culicidae)*. Ph. D. thesis, University of Valencia, Valencia.
- Bueno-Marí, R., Bernués-Bañeres, A., and Jiménez-Peydró, R. (2012). Updated checklist and distribution maps of mosquitoes (Diptera: Culicidae) of Spain. *Eur. Mosq. Bull.* 30, 91–126.
- Burkett-Cadena, N. D., McClure, C. J. W., Estep, L. K., and Eubanks, M. D. (2013). Hosts or habitats: what drives the spatial distribution of mosquitoes? *Ecosphere* 4:30.
- Calzolari, M. (2016). Mosquito-borne diseases in Europe: an emerging public health threat. *Rep. Parasitol.* 5, 1–12. doi: 10.2147/RIP.S56780
- de Valdez, M. R. W. (2017). Mosquito species distribution across urban, suburban, and semi-rural residences in San Antonio, Texas. *J. Vect. Ecol.* 42, 184–188. doi: 10.1111/jvec.12254
- Gobierno Vasco-Eusko Jaurlaritz (2019). *Estudio Comercial Vitoria-Gasteiz*. Available online at: <http://www.comercio.vitoria.com/wp-content/uploads/2019/02/Estudio-Comercial-Vitoria-Gasteiz.pdf> (accessed March 18, 2020).
- Eritja, R., Escosa, R., Lucientes, J., Marqués, E., Roiz, D., and Ruiz, S. (2005). Worldwide invasion of vector mosquitoes: present European distribution and challenges for Spain. *Biol. Invas.* 7:87. doi: 10.1007/s10530-004-9637-6
- Eritja, R., Ruiz-Arrondo, I., Delacour-Estrella, S., Schaffner, F., Álvarez-Chachero, J., Bengoa, M., et al. (2019). First detection of *Aedes japonicus* in Spain: an unexpected finding triggered by citizen science. *Parasit. Vect.* 12:53. doi: 10.1186/s13071-019-3317-y
- Failloux, A. B., Bouattour, A., Faraj, C., Gunay, F., Haddad, N., Harratet, Z., et al. (2017). Surveillance of arthropod-borne viruses and their vectors in the Mediterranean and Black Sea regions within the MediLabSecure network. *Curr. Trop. Med. Rep.* 4, 27–39. doi: 10.1007/s40475-017-0101-y
- Ferraguti, M., Martínez-de la Puente, J., Roiz, D., Ruiz, S., Soriguer, R. C., and Figuerola, J. (2016). Effects of landscape anthropization on mosquito community composition and abundance. *Sci. Rep.* 6:29002. doi: 10.1038/srep29002
- Goiri, F., González, M. A., Goikolea, J., Oribe, M., de Castro, V., Delacour, S., et al. (2020). Progressive invasion of *Aedes albopictus* in Northern Spain in the period 2013–2018 and a possible association with the increase in insect bites. *Int. J. Environ. Res. Public Health* 17:1678. doi: 10.3390/ijerph17051678
- Gomes, B., Sousa, C. A., Vicente, J. L., Pinho, L., Calderón, I., Arez, E., et al. (2013). Feeding patterns of *molestus* and *pipiens* forms of *Culex pipiens* (Diptera: Culicidae) in a region of high hybridization. *Parasit. Vect.* 6:93. doi: 10.1186/1756-3305-6-93
- González, M., Goiri, F., Barandika, J. F., and García-Pérez, A. L. (2020). *Culicoides* biting midges and mosquito fauna at three dog and cat shelters in rural and periurban areas in Northern Spain. *Med. Vet. Entomol.* 25:mve.12471. doi: 10.1111/mve.12471
- González, M., López, S., and Alarcón-Elbal, P. M. (2015). Blood-feeding Diptera (Culicidae and Ceratopogonidae) in an urban park of the city of Vitoria-Gasteiz (Basque Country, Spain). *J. Eur. Mosq. Control Assoc.* 33, 10–14.
- Gutiérrez-López, R., Martínez-de la Puente, J., Gangoso, L., Yan, J., Soriguer, R. C., and Figuerola, J. (2016). Do mosquitoes transmit the avian malaria-like parasite *Haemoproteus*? An experimental test of vector competence using mosquito saliva. *Parasit. Vect.* 9:609. doi: 10.1186/s13071-016-1903-9
- Hajibabaei, M., Smith, M. A., Janzen, D. H., Rodríguez, J. J., Whitfield, J. B., and Hebert, P. D. N. (2006). A minimalist barcode can identify a specimen whose DNA is degraded. *Mol. Ecol. Notes* 6, 959–964. doi: 10.1111/j.1471-8286.2006.01470.x
- Hebert, P. D., Cywinska, A., Ball, S. L., and de Waard, J. R. (2003). Biological identifications through DNA barcodes. *Proc. Biol. Sci.* 270, 313–321. doi: 10.1098/rspb.2002.2218
- Hernández-Triana, L. M., Prosser, S. W., Rodríguez-Pérez, M. A., Chaverri, L. G., Hebert, P. D., and Gregory, T. R. (2014). Recovery of DNA barcodes from blackfly museum specimens (Diptera: Simuliidae) using primer sets that target a variety of sequence lengths. *Mol. Ecol. Resour.* 14, 508–518. doi: 10.1111/1755-0998.12208
- Honnen, A. C., and Monaghan, M. T. (2017). City-Dwellers and country folks: lack of population differentiation along an urban-rural gradient in the mosquito *Culex pipiens* (Diptera: Culicidae). *J. Insect. Sci.* 17:107. doi: 10.1093/jisesa/iex086
- Ibañez-Justicia, A., Stroo, A., Dik, M., Beeuwkes, J., and Scholte, E. J. (2015). National mosquito (Diptera: Culicidae) survey in The Netherlands 2010–2013. *J. Med. Entomol.* 52, 185–198. doi: 10.1093/jme/tju058
- Ivanova, N. V., de Waard, J., and Hebert, P. D. N. (2006). An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Mol. Ecol. Notes* 6, 998–1002. doi: 10.1111/j.1471-8286.2006.01428.x
- Kampen, H., Schäfer, M., Zielke, D. E., and Walther, D. (2016). The *Anopheles maculipennis* complex (Diptera: Culicidae) in Germany: an update following recent monitoring activities. *Parasitol. Res.* 115, 3281–3294. doi: 10.1007/s00436-016-5189-9
- Kilpatrick, A. M., LaDeau, S. L., and Marra, P. P. (2007). Ecology of West Nile virus transmission and its impact on birds in the western hemisphere. *Auk* 124, 1121–1136. doi: 10.1093/auk/124.4.1121
- Kothera, L., Mutebi, J. P., Kenney, J. L., Saxton-Shaw, K., Ward, M. P., and Savage, H. M. (2020). Bloodmeal, host selection, and genetic admixture analyses of *Culex pipiens* complex (Diptera: Culicidae) mosquitoes in Chicago, IL. *J. Med. Entomol.* 57, 78–87. doi: 10.1093/jme/tjz158
- Leishnam, P. T., LaDeau, S. L., and Juliano, S. A. (2014). Spatial and temporal habitat segregation of mosquitoes in urban Florida. *PLoS One* 9:e91655. doi: 10.1371/journal.pone.0091655
- Li, Y., Kamara, F., Zhou, G., Puthiyakunnon, S., Li, C., Liu, Y., et al. (2014). Urbanization increases *Aedes albopictus* larval habitats and accelerates mosquito development and survivorship. *PLoS Negl. Trop. Dis.* 8:e33301. doi: 10.1371/journal.pntd.0003301
- Maia, M. F., Robinson, A., John, A., Mgando, J., Simfukwe, E., and Moore, S. J. (2011). Comparison of the CDC Backpack aspirator and the Prokopack aspirator for sampling indoor- and outdoor-resting mosquitoes in Southern Tanzania. *Parasit. Vect.* 4:124. doi: 10.1186/1756-3305-4-124
- Martinet, J. P., Ferté, H., Failloux, A. B., Schaffner, F., and Depaquit, J. (2019). Mosquitoes of North-Western Europe as potential vectors of arboviruses: a review. *Viruses* 11:1059. doi: 10.3390/v11111059

- Martínez-de la Puente, J., Ferraguti, M., Ruiz, S., Roiz, D., Soriguer, R. C., and Figuerola, J. (2016). *Culex pipiens* forms and urbanization: effects on blood feeding sources and transmission of avian *Plasmodium*. *Malar. J.* 15:589. doi: 10.1186/s12936-016-1643-5
- Martínez-de la Puente, J., Ruiz, S., Soriguer, R., and Figuerola, J. (2013). Effect of blood meal digestion and DNA extraction protocol on the success of blood meal source determination in the malaria vector *Anopheles atroparvus*. *Malar. J.* 12:109. doi: 10.1186/1475-2875-12-109
- McDonnell, M. J., and Pickett, S. T. (1990). Ecosystem structure and function along urban-rural gradients: an unexploited opportunity for ecology. *Ecology* 71, 1232–1237. doi: 10.2307/1938259
- Medlock, J. M., and Vaux, A. G. (2015). Impacts of the creation, expansion and management of English wetlands on mosquito presence and abundance - developing strategies for future disease mitigation. *Parasit. Vect.* 8:142. doi: 10.1186/s13071-015-0751-3
- Möhlmann, T. W. R., Wennergren, U., Tälle, M., Favia, G., Damiani, C., Bracchetti, L., et al. (2017). Community analysis of the abundance and diversity of mosquito species (Diptera: Culicidae) in three European countries at different latitudes. *Parasit. Vect.* 10:510. doi: 10.1186/s13071-017-2481-1
- Muñoz, J., Eritja, R., Alcaide, M., Montalvo, T., Soriguer, R. C., and Figuerola, J. (2011). Host-feeding patterns of native *Culex pipiens* and invasive *Aedes albopictus* mosquitoes (Diptera: Culicidae) in urban zones from Barcelona, Spain. *J. Med. Entomol.* 48, 956–960. doi: 10.1603/me11016
- Muñoz, J., Ruiz, S., Soriguer, R., Alcaide, M., Viana, D. S., Roiz, D., et al. (2012). Feeding patterns of potential West Nile virus vectors in South-West Spain. *PLoS One* 7:e39549. doi: 10.1371/journal.pone.0039549
- Overgaard, H. J., Ekbohm, B., Suwonkerd, W., and Takagi, M. (2003). Effect of landscape structure on anopheline mosquito density and diversity in Northern Thailand: implications for malaria transmission and control. *Landsc. Ecol.* 18:605. doi: 10.1023/A:1026074910038
- Platt, K. B., Tucker, B. J., Halbur, P. G., Tiawirisup, S., Blitvich, B. J., Fabiosa, F. G., et al. (2007). West Nile virus viremia in eastern chipmunks (*Tamias striatus*) sufficient for infecting different mosquitoes. *Emerg. Infect. Dis.* 13, 831–837. doi: 10.3201/eid1306.061008
- Radrova, J., Seblova, V., and Votypka, J. (2013). Feeding behavior and spatial distribution of *Culex* mosquitoes (Diptera: Culicidae) in wetland areas of the Czech Republic. *J. Med. Entomol.* 50, 1097–1104. doi: 10.1603/me13029
- Rizzoli, A., Bolzoni, L., Chadwick, E. A., Capelli, G., Montarsi, F., Grisentie, M., et al. (2015). Understanding West Nile virus ecology in Europe: *Culex pipiens* host feeding preference in a hotspot of virus emergence. *Parasit. Vect.* 8:213. doi: 10.1186/s13071-015-0831-4
- Roiz, D., Vazquez, A., Rosa, R., Muñoz, J., Arnoldi, D., Rosso, F., et al. (2012). Blood meal analysis, flavivirus screening, and influence of meteorological variables on the dynamics of potential mosquito vectors of West Nile virus in Northern Italy. *J. Vect. Ecol.* 37, 20–28. doi: 10.1111/j.1948-7134.2012.00196.x
- Ruiz-Arrondo, I., Hernández-Triana, L. M., Nikolova, N. I., Fooks, A. R., and Oteo, J. A. (2020). Integrated approaches in support of taxonomic identification of mosquitoes (Diptera: Culicidae) in vector surveillance in Spain. *Vect. Borne Zoonot. Dis.* doi: 10.1089/vbz.2020.2662 [Epub ahead of print].
- Ruiz-Arrondo, I., McMahon, B. J., Hernández-Triana, L. M., Santibañez, P., Portillo, A., and Oteo, J. A. (2019). Surveillance of mosquitoes (Diptera, Culicidae) in a Northern Central region of Spain: implications for the medical community. *Front. Vet. Sci.* 6:86. doi: 10.3389/fvets.2019.00086
- Santos, C. S., Pie, M. R., da Rocha, T. C., and Navarro-Silva, M. A. (2019). Molecular identification of blood meals in mosquitoes (Diptera, Culicidae) in urban and forested habitats in Southern Brazil. *PLoS One* 14:e0212517. doi: 10.1371/journal.pone.0212517
- Schaffner, F., Angel, G., Geoffroy, B., Hervy, J. O., and Rhaeim, A. (2001). *The Mosquitoes of Europe. IRD Éditions and EID Méditerranée*. Montpellier: CD-Rom.
- Shaikovich, E. V., Vinogradova, E. B., Bouattour, A., and Gouveia de Almeida, A. N. P. (2016). Genetic diversity of *Culex pipiens* mosquitoes in distinct populations from Europe: contribution of *Cx. quinquefasciatus* in Mediterranean populations. *Parasit. Vect.* 9:47. doi: 10.1186/s13071-016-1333-8
- Silver, J. B. (2008). *Mosquito Ecology: Field Sampling Methods*, 3rd Edn, New York, NY: Springer.
- Vázquez, A., Ruiz, S., Herrero, L., Moreno, J., Molero, F., Magallanes, A., et al. (2011). West Nile and Usutu viruses in mosquitoes in Spain, 2008–2009. *Am. J. Trop. Med. Hyg.* 85, 178–181. doi: 10.4269/ajtmh.2011.11-0042
- Vazquez-Prokopec, G. M., Galvin, W. A., Kelly, R., and Kitron, U. (2009). A new, cost-effective, battery-powered aspirator for adult mosquito collections. *J. Med. Entomol.* 46, 1256–1259. doi: 10.1089/vbz.2018.2381
- Vicente, J. L., Sousa, C. A., Alten, B., Caglar, S. S., Falcutá, E., Latorre, J. M., et al. (2011). Genetic and phenotypic variation of the malaria vector *Anopheles atroparvus* in Southern Europe. *Malar. J.* 10:5. doi: 10.1186/1475-2875-10-5

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 González, Prosser, Hernández-Triana, Alarcón-Elbal, Goiri, López, Ruiz-Arrondo, Hebert and García-Pérez. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.